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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER P64029US0
		US APPLICATION NO. (if known, see 37 CFR 1.5) 09/423622
INTERNATIONAL APPLICATION NO. PCT/EP98/02808	INTERNATIONAL FILING DATE 13 May 1998	PRIORITY DATE CLAIMED 14 May 1997

TITLE OF INVENTION

A METHOD FOR THE IMPROVEMENT OF NEURONAL REGENERATION

APPLICANT(S) FOR DO/EO/US

Prof. H. W. MÜLLER and Christine C. STICHEL-GUNKEL

Applicant herein submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for Internatl. Preliminary Examination was made by the 19th month from earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ A translation of the annexes to the Internatl. Preliminary Examination report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    PCT/IB/304 Form
    PCT/IB/308 Form
    First Page of Publication
    International Preliminary Examination Report (w/Annexes)

US APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/423622	PCT/EP98/02808	P64029US0

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Internatl. prelim. examination fee paid to USPTO (37 CFR 1.492 (a) (1)) .. $670.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (2)) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .. $760.00		
Neither international preliminary examination fee (37 CFR 1.492 (a) (3)) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO) **$970.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (4)) and all claims satisfied provisions of PCT Article 33(2)-(4) $96.00		
Search Report prepared by the EPO or JPO (37 CFR 1.492 (a) (5)) **$840.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of $130.00 for furnishing the **oath or declaration** later than ☐ **20** ☒ **30** months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	17 - 20 =	-0-	x $18.00	$	
Independent Claims	3 - 3 =	-0-	x $78.00	$	
Multiple Dependent Claim(s) (if applicable)			+ $260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 970.00	
Reduction by 1/2 for filing by **small entity**, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$ 970.00	
Processing fee of $130 for furnishing the **English translation** later than ☐ **20** ☐ **30** months from the earliest claimed priority date (37 CFR 1.492(f))				$	
TOTAL NATIONAL FEE =				$ 970.00	
Fee of $40.00 for recording the enclosed **assignment** (37 CFR 1.21(h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31).				$	
TOTAL FEES ENCLOSED =				$ 970.00	
				Amt. to be refunded:	$
				Amt. charged:	$

a. ☒ A check in the amount of $ 970.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. **06-1358** in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge my account any additional fees set forth in §1.492 during the pendency of this application, or credit any overpayment to Deposit Account No. **06-1358**. A duplicate copy of this sheet is enclosed.

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By _____
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JPH&S 3/95

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Prof. H. W. MÜLLER et al.

Serial No.: New

Filed: November 15, 1999

For: A METHOD FOR THE IMPROVEMENT OF NEURONAL REGENERATION

PRELIMINARY AMENDMENT TO LESSEN FEES

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE CLAIMS

Claim 4, line 1, delete "claims 2 and/or 3",
insert --claim 2--;
Claim 6, line 1, delete "any one of the preceding claims",
insert --claim 1--;
Claim 7, line 1, delete "any one of the preceding claims",
insert --claim 1--;
Claim 8, line 1, delete "any one of the preceding claims",
insert --claim 1--;
Claim 9, line 1, delete "any one of the preceding claims",
insert --claim 1--;
Claim 13, line 1, delete "claims 10 or 11",
insert --claim 10--;
Claim 17, line 1, delete "or 16".

REMARKS

The foregoing Preliminary Amendment is requested in order to delete the multiple dependent claims and avoid paying the multiple dependent claims fee.

Early action on the merits is respectfully requested.

Respectfully submitted,

JACOBSON, PRICE, HOLMAN & STERN, PLLC

By _____
William E. Player
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Date: November 15, 1999
Atty. Docket: P64029US0
WEP:crj

Law Offices of
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PROFESSIONAL LIMITED LIABILITY COMPANY
THE JENIFER BUILDING
400 SEVENTH STREET, N.W.
WASHINGTON, DC 20004

Attny's Docket No. P64029US0

SMALL ENTITY DECLARATION
[37 CFR 1.9(c-f)]

Each undersigned declares that:

(1) ☐ the application attached hereto.

(2) ☒ U.S. Application Serial No. _____, filed November 15, 1999

(3) ☐ U.S. Patent No. _____ Issued _____

is entitled to the benefits of "small entity" status for paying reduced fees under 35 USC 41(a) and (b) to the Patent and Trademark Office by virtue of the following:

(4) ☒ Each undersigned declares that he/she qualifies as an independent inventor, or would qualify had he/she made the as defined in 37 CFR 1.9(c).

(5) ☐ The undersigned declares that he/she is an official empowered to act on behalf of the concern identified below; that concern qualifies as a small business concern as defined in 37 CFR 1.9(d); that exclusive rights to the invention have been conveyed to and remain with the small business concern, or if the rights are not exclusive, that all other rights belong to small entities as defined in 37 CFR 1.9.

(6) ☐ The undersigned declares that he/she is an official empowered to act on behalf of the organization identified below; that organization qualifies as a nonprofit organization as defined in

(a) ☐ 37 CFR 1.9(e)(1)

(b) ☐ 37 CFR 1.9(e)(2)

(c) ☐ 37 CFR 1.9(e)(3)

(d) ☐ 37 CFR 1.9(e)(4) State law of _____ ;

that exclusive rights to the invention have been conveyed to and remain with the organization, or if the rights are not exclusive, that all other rights belong to organizations as defined in 37 CFR 1.9.

(7) Each person, concern or organization to which I/we have assigned, granted, conveyed or licensed, or am under an under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

(a) ☒ no such person, concern or organization

(b) ☐ persons, concerns or organization listed below

[a separate declaration is required from *each* named person, concern or organization having rights to this invention averring to their status as "small entities."]

Full Name _____

Address _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

I/we acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement of small entity prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I/we hereby declare all statements made herein of his/her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application, any patent issued thereon, or any patent to which this declaration is directed.

(8) **H. W. MÜLLER** — Typed Name of Inventor — Signature: Hans W. Müller — Date: Dec 22, 1999

Christine C. STICHEL-GUNKEL — Typed Name of Inventor — Signature: Chr. Stichel-Gunkel — Date: 24.12.1999

_____ — Typed Name of Inventor — Signature _____ — Date _____

_____ — Typed Name of Inventor — Signature _____ — Date _____

(9) _____
Name of Small Business Concern or Nonprofit Organization

_____ By _____ — Typed Name — Signature — Date

_____
Title of Signatory

A method for the improvement of neuronal regeneration

The present invention refers to a method for the improvement of neuronal regeneration, a medicament for the improvement of neuronal regeneration and use of a specific inhibitor substance.

Injury to adult mammalian CNS fiber tracts leads to the formation of a lesion scar consisting of a convoluted fringe of astroglial processes lined by a basal membrane (BM). This lesion scar is implicated as a major extrinsic constraint to effective axon regeneration in brain and spinal cord (1 - 4). While the dense astrocytic network is a permissive substrate for axon growth (5, 6), the presence of BM has been hypothesized as a crucial impediment for regeneration (7). However, experimental evidence was not shown. To the contrary, when the BM formed after a lesion of neuronal tissue was removed (24), no improved regeneration could be reproducibly monitored (25). Therefore, it is still of great importance to have a method for improving regeneration of injured neurons.

WO 93/19783 discloses a method for preventing, supressing or treating a CNS pathology characterized by a deleterious accumulation of extracellular matrix in a tissue by contacting the tissue with an agent that inhibits the extracellular matrix producing activity of TGF-ß. The disclosed methods can be used to prevent, suppress or treat scar formation in the CNS. As useful agents are addressed neutralizing anti-TGF-ß antibodies, Arg-Gly-Asp-containing peptides, decorin and its functional equivalence such as biglycan and TGF-ß antagonists. TGF-ß has a wide spectrum of physiological functions such as activation of cell of the immune system, inhibition of cell proliferation, neurotrophic effects on sensory neurons, inhibition of Schwann cell myelination, anti-profilerative effects on glial cells,

immunsuppressive effects, stimulation of extracellular matrix deposition and chemoattraction of microglia cells. The anti-TGF-ß treatment would induce the opposite effects. Inhibition of TGF-ß activity leads to numerous non-specific cellular responses, which may even lead to unwanted side effects. One object of the invention is to avoid such potential unwanted side effects.

Surprisingly, improvement of regeneration of neuronal tissue after lesion is achieved by a method of the present invention.

According to the method of the invention improved regeneration of injured neuronal tissue is achieved by specific prevention or specific inhibition of basal membrane formation induced by a lesion of neuronal tissue.

The basal membrane is a structure which is composed of different elements. Elements of the basal membrane are collagen IV, laminin, entactin (Nidogen) accessory substances. The assembly of the elements to the basal membrane is performed by enzymes which may be assisted by cofactors.

Inhibitors of TGF-ß are not involved with a specific prevention or specific inhibition of basal membrane formation induced by lesion of neuronal tissue. According to the present invention it is achieved in an advantageous manner that a specific interaction is provided.

Preferably, the formation of the basal membrane is prevented or inhibited by applying a specific inhibitor substance of the synthesis of basal membrane building elements, or the assembly of basal membrane building elements, or both the synthesis of basal membrane building elements and the assembly of basal membrane building elements to a body in need thereof. The building elements of the basal membrane are in particular those which are involved with the formation of the basal membrane, for instance molecular structures building up the basal membra-

ne, such as monomeric compounds, accessory substances, substances for the assembly of the components of the basal membrane and the like.

In particular, the basal membrane building elements are selected from the group consisting of collagen IV, laminin, entactin, accessory substances for proper function, or the assembly of the basal membrane, or both the proper function and the assembly of the basal membrane.

A specific inhibitor substance of the invention is capable of preventing or inhibiting the formation of the basal membrane and/or is specifically interfering with the assembly process of the basal membrane. Preferably, the specific inhibitor substance is selected from the group consisting of antibodies against collagen IV, laminin, entactin, accessory substances for proper function, or the assembly of the basal membrane; Fe-chelating agents; inhibitors of amino acids hydroxylases, such as prolyl-4-hydroxylase, lysine-hydroxylase; 2-oxoglutarate competitors; antisense nucleotides or nucleotide analogs which are able to prevent or inhibit the expression of basal membrane building elements, and the like.

According to the invention can further be used those inhibitor substances which are selected from the group consisting of N-oxaloglycine; Zn salts; pyridine derivatives, such as 5-arylcarbonyamino- or 5-arylcarbamoyl- derivatives, 2-carboxylate, 2,5 dicarboxylate, their ethyl esters or ethyl amides or -5-acyl sulfonamides, 2,4 dicarboxylate, their ethyl esters or ethylamides, or dimethoxyethylamides; 3,4 bipyridine, such as 5 amino-6-(1H)-one, 1,6-dihydro-2-methyl-6-oxo-5-carbonitril; 2,2'-bipyridine, such as 5,5'-dicarboxylic acid or its pharmaceutically acceptable salts, 4,4'-dicarboxylic acid ethyl ester or ethyl amide; 3,4'-dihydroxybenzoate, such as the diethyl ester; proline and its structural and functional analoges; ß-aminopropionitrile; desferrioxamine; anthracyclines; 2,7,8-trihydroxy anthraquinones, fibrostatin-C; coumalic acid or its pharmaceuti-

cally acceptable salts; 5-oxaproline,ß-lactam antibiotics.

In a preferred embodiment of the present invention the specific inhibitor substance(s) are applied in combination with one or more substances being capable of stimulating neuronal growth or inducing the expression of growth promoting proteins. Such neuronal growth stimulating substances are neurotrophic growth factors of the neurotrophin family and other growth factor families such as fibroblast growth factors, insulin and insulin-like growth factors, as well as epidermal growth factor, ciliary neuronotrophic growth factor (CNTF), glial cell-derived growth factor (GDNF), cytokines, neurotrophic proteoglycans and glycosamino-glycans, neural cell adhesion molecules like L1 (NILE), growth-associated proteins like GAP43 and anti-apoptotic proteins like bcl-2.

According to the invention it is preferred to locally apply the specific inhibitor substances in the neuronal tissue, intraventricularly, or systemically, in particular orally or intravenously.

The concentration of the specific inhibitor substance varies in view of the chemical nature. For example, antisense inhibitor substances may have more specific effects so that lesser amounts can be applied.

Typically, the specific inhibitor substance is applied in therapeutically effective amounts, such as 1 ng/kg to 1 mg/kg body weight, when low molecular compounds such as bipyridyl-derivatives are applied.

The invention also provides a medicament for the improvement of neuronal regeneration comprising a therapeutically effective amount of a specific inhibitor substance which is capable of prevention or inhibition of basal membrane formation induced by a lesion of neuronal tissue. Appropriate specific inhibitor substances are described hereinabove. The medicament may further

comprise carrier substances or adjuvants in order to facilitate an appropriate application. The medicament may further comprise substances which are capable of stimulating neuronal growth.

The specific inhibitor substances which are capable of prevention or inhibition of basal membrane formation induced by a lesion of neuronal tissue can be used for the manufacturing of a medicament of the invention.

Fig. 1:
Expression of collagen IV and axonal sprouting after transection of the postcommissural fornix in untreated animals (b-d) and after injection of anti-Coll IV (e) or DPY (f) at two weeks postsurgery. a, Sagittal view of the adult rat brain showing the course of the fornix and the location of the transection site. Marked deposition of collagen IV in the lesion site (arrow) and proximal stump (P) of an untreated animal at low (b) and high magnification (c). Note, the fine structure and the spatial orientation of collagen IV deposits perpendicular to the trajectory of the tract. d, In untreated animals regrowing fornix axons stop sharply at the lesion site (arrow). Collagen IV deposition is markedly reduced in the lesion site after anti-Coll IV (e) or DPY injection (f). Scale bars, 100 µm.

Fig. 2: Regeneration of transected fornix fibers across the lesion site in rats treated with anti-Coll IV (a, c, e) or DPY (b, d, f) at 6 weeks postsurgery. Sagittal serial sections reacted for NF-immunohistochemistry show that in both experimental groups fibers traverse the former lesion site (arrows) (c, d) and elongate within the distal stump (e, f) up to the mammillary body (MB). Scale bars, 100 µm.

Fig. 3: Recovery of structural features of the regenerating fornix tract. a, b Anterograde tracing with biocytin of an anti-Coll IV treated animal at 6 weeks postsurgery reveals the large number of regenerating axons (a), their elongation within the former pathway (a) and their fine varicose morphology (b). c,

Large WGA-HRP-filled axon (arrowhead) in the mammillary body surrounded by compact myelin (arrows). d, e Electron micrographs of anterogradely WGA-HRP-labeled presynaptic terminals (arrowheads) in the mammillary body at 6 weeks after anti-Coll IV treatment. Scale bars, 100 μm (a), 50 μm (b), 0.1 μm (c), 0.5 μm (d), 1 μm (e).

Fig. 4: Electrophysiological properties of fornix fibers in unlesioned rats and lesioned/injected animals with regeneration. a, Schematic illustration showing the location of the stimulating (S) and recording (R) electrode at various conditions. b, Characteristic recordings of extracellular action potentials in a sagittal slice prepared from an animal with regeneration. Recordings were obtained under conditions as illustrated in a. Application of Tetrodotoxin (TTX) blocks the stimulus-evoked response. The net action potential is shown in trace 5. c and d, Distribution of conduction velocity and action potential response amplitude in unlesioned and lesioned/injected animals with regeneration.

The mechanically transected postcommissural fornix of the adult rat, a unidirectional and well-characterized fiber tract (8,9), was used to determine whether specific biochemical or immunochemical modulation of BM formation would provide a means to stimulate axon regeneration. Here we report that lesion-induced BM deposition can be significantly reduced by local injection of anti-collagen IV antibodies or α,α dipyridyl, an inhibitor of collagen triple helix formation and synthesis. Reducing the collagen network allowed massive axon elongation across the lesion site. The regenerating fornix fibers followed the original pathway, reinnervated their appropriate target, the mammillary body, were remyelinated and attained nearly normal conduction properties. on failure of adult mammalian CNS axons we examined its spatio-temporal distribution pattern after penetrant CNS lesion and determined whether its remodelling allows structural and functional regeneration of a transected CNS fiber tract.

The left postcommissural fornix was stereotactically transected in adult Wistar rats Fig. 1a) and the postlesion deposition of BM was analyzed using antibodies against collagen IV (Coll IV) and laminin (LN), the major and unique components of BM (10,11). By the end of the second week after lesion the center of the wound was filled by Coll IV- and LN-rich BM (Fig. 1b, c). These newly formed BM were either arranged in long continuous layers or associated with numerous blood vessels. Within the center of the wound the BM layers formed a parallel array aligned perpendicular to the course of the fiber tract (Fig. 1b, c). In the vicinity of the transected stumps, however, BM layers were deposited as hook-like turns extending along the longitudinal tract axis for about 200 µm into the fornix stumps (Fig. 1c). In parallel with the deposition of the BM, sprouting axons in the proximal stump reached the lesion site. They failed to cross or bypass it but stopped growing at the wound border at about 2 weeks after lesion (Fig. 1d). The spatio-temporal coincidence of BM formation with the abrupt axonal growth arrest at the tract-lesion border strongly suggests that the newly formed perpendicular layers of BM could be a physical impediment for regenerating axons.

In an effort to modulate postlesion BM deposition, either polyclonal antibodies against collagen IV (anti-Coll IV; n=14) or the iron chelator a, a'-dipyridyl (DPY; n=9) were injected locally into the lesion center immediately after transection. DPY is a competitive inhibitor of prolyl 4-hydroxylase (12) and has been shown to prevent collagen triple helix formation (12), which results in feedback inhibition of procollagen synthesis (13) and enhanced procollagen degradation (14). Control animals received a PBS injection (n=9) or were sham operated (n=3). Basal membrane formation was studied in response to antibody and drug treatment using immunohistochemical methods. Animals receiving a single injection of anti-Coll IV (80-160 ng) or DPY (1.6-16 µmol) showed a massive and specific reduction in Coll-immunopositive laminae and blood vessels in the lesion center and the fornix stumps at all examined survival time points. At

2 weeks after lesion+injection only a very small number of Coll-immunreactive structures perpendicular to the tract course had developed (Fig. 1e, f). Control animals, however, exhibited dense BM deposition as previously described for lesion only animals. The applied substances reduced the deposition of BM at the lesion site but did not affect the number or the distribution of vascular BM in the surrounding neuropil. Therefore, we conclude that the lesion-induced BM formation can be specifically reduced by immediate application of either anti-Coll IV antibodies or DPY.

To determine whether reduction of BM deposition would permit regeneration of transected axons across the lesion site, we studied the elongation of fornix axons after anti-Coll IV or DPY treatment using immunocytochemical staining. While sprouting fornix fibers in control animals ceased growing at the proximal stump-lesion interface (Fig. 1d) large numbers of axons entered and traversed the lesion center between 2 and 4 weeks after lesion+injection in those animals receiving anti-Coll IV (n= 11) (Fig. 2 a, c, e) or DPY treatment (n= 6) (Fig. 2 b, d, f). Most regenerating axons formed a loop over the lesion site, entered the distal stump and continued in a parallel bundle of fine and beaded axons within their previous pathway (Fig. 3a, b). They reached their appropriate target, the mammillary body, at about 4-6 weeks postsurgery. Anterograde tracing with WGA-HRP into the subiculum, the origin of the fornix (not shown), or biocytin application into the proximal fornix stump (Fig. 3a) provided proof, that the vast majority of fibers emerge from the formerly transected fornix tract. All regenerating fornix axons remained within their original pathway and did not invade the surrounding neuropil. The present results demonstrate that the failure of postcommissural fornix regeneration in rat brain, in fact, depends upon the formation of an axon growth-inhibiting BM at the lesion site that is oriented perpendicular to the tract course. Reduction of BM deposition seems to be a prerequisite but also a sufficient condition for the transected axons to regenerate across the lesion site.

Further preferred embodiments for restitution of functional circuitry after traumatic CNS lesion are the remyelination of regenerated fibers, the re-establishment of synaptic connections with the appropriate target and the restoration of normal conduction properties. Structural and functional properties of the regenerating axons were investigated using immunohistochemical, morphological and electrophysiological methods. Immunohistochemistry with an antibody against myelin basic protein demonstrated the remyelination of regenerated fornix axons along their entire length as early as 4 weeks after surgery (data not shown). This observation was confirmed by ultrastructural analysis of anterogradely WGA-HRP labeled axons in the distal stump which showed clear evidence of compact myelin sheath formation (Fig. 3c). In addition, ultrastructural studies provided evidence for the re-establishment of synaptic connections of regenerating axons within the mammillary body. Tracer reaction product was identified in presynaptic profiles with round vesicles that formed asymmetric synaptic junctions at unlabeled dendrites (Fig. 3d, e). The ultrastructural features of the labelled presynaptic profiles correspond to those described for the RA-type (round, asymmetric) of synaptic terminal, which is considered to be of subicular origin (8). The electrophysiological properties of regenerated fibers were studied using extracellular in vitro recording techniques applied to sagittal brain slices (400 μm) of 8 unlesioned rats and 4 treated animals showing regenerated fiber tracts. In unlesioned animals electrical stimulation of the fornix fibers elicited an extracellular action potential with an amplitude of 1.02 $\pm$ 0.14 mV and a conduction velocity of 0.48 $\pm$ 0.05 m/s (mean $\pm$ SEM, n=16, Fig. 4b-d). This axonal conduction velocity corresponds well to previously reported measurements (about 0.5 m/s for hippocampal Schaffer collaterals (15). Similar values for action potential amplitude and conduction velocity (1.12 $\pm$ 0.21 mV, 0.46 $\pm$ 0.1 m/s, n=5) were obtained in regenerating animals when the stimulating (S) and the recording (R) electrodes were positioned proximally to the lesion site (see S1 and R1 in Fig. 4a). In the latter animals, functionally intact fibers showing

normal extracellular action potential amplitude and conduction velocity could also be demonstrated across (S3 and R3 in Fig. 4a; 0.8 ± 0.29 mV, 0.54 ± 0.14 m/s, n=3) and distal to the lesion site (S2 and R2 in Fig. 4a; 0.91 ± 0.24 mV, 0.43 ± 0.06 m/s, n=4) (Fig. 4c, d). In all animals, the stimulus-evoked extracellular responses were blocked by Tetrodotoxin, confirming their nature as Na+-dependent action potentials (Fig. 4b). From these data we conclude that the reorganization of the fornix tract is accompanied by structural and functional recovery of the regenerated axons.

Our results demonstrate that structural and functional restoration of lesioned mature fornix pathway can be achieved by reduction of BM formation in the lesion site. Data described here underscore the importance of extrinsic determinants in axonal regeneration but also demonstrates that once the axons have crossed the lesion scar other potential extrinsic regeneration constraints, like CNS myelin and oligodendrocytes (9,16-18), dense astrogliosis (6) and sulfated proteoglycans (19,20), do not impede their progress. The results further indicate that similar to other CNS circuits (21,22), fornix axons have an innate potential for regeneration and self-organization. These results give rise to new and promising concepts for therapeutic strategies that might contribute to the reduction of neurological deficits after CNS lesions.

The following examples are intended for further illustration of the invention but are not limiting.

Surgery. The left postcommissural fornix of 42 Wistar rats (180-210g) was transected stereotactically at a distance of about 1 mm proximal to the target, the mammillary body, using a Scouten wire knife as described previously (9). The completeness of transection was confirmed by serial reconstruction of the lesion site for each of the animals. Immediately after transection animals received a topical application (1.6 µl) of either polyclonal antibodies against collagen IV (anti-Coll IV, Bioge-

nex, 50-100 µg/ml, n=14) or the iron chelator a, a'-dipyridyl (DPY, 1-10 mM, n=9). Substances were pressure injected (injection time 10 min) directly into the lesion site via a micropipette coupled to a microsyringe. Controls received equal amounts of phosphate-buffered saline (n=9) or sham operation (n=3).

Anterograde tracing was performed for analysis of fiber course, ultrastructural morphology and target reinnervation. After a survival time of 6 weeks, anti-Coll IV-treated animals (n=4) received two injections of a 2% (w/v) solution of wheat-germ-agglutinin-HRP (WGA-HRP) into the left subicular complex (dorsal and caudal pole). Rats were perfused 3 days later with 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer. Vibratome sections were reacted for WGA-HRP using tetramethylbenzidine as substrate (23).

Electron microscopy. For ultrastructural analysis vibratome sections of anti-Coll IV-treated animals were reacted for WGA-HRP, immersed for 12h in 1% osmium tetroxide and embedded in epon. Ultrathin sections were examined using a Hitachi H600 electron microscope.

Immunohistochemical staining. After a survival time of 4 days (d), 6d, 2 weeks (w), 4w and 6w after surgery brains were removed, frozen in isopentan (-50/-60°C) and cut into serial sagittal 10 µm thick sections. Sections were fixed with acetone (-20°C), preincubated in 3% H2O2 (v/v) in methanol to block endogeneous peroxidase, followed by PBS containing 3% (v/v) normal horse or normal goat serum to reduce unspecific staining and then incubated with one of the following primary antibodies: polyclonal anti-collagen IV (anti-Coll IV, Biogenex, 1:3), polyclonal anti-laminin (anti-LN, Biogenex, 1:5) or monoclonal cocktail against phosphorylated neurofilaments (anti-NF, Affinity, 1:800). Following, avidin-biotin-peroxidase complex staining (Vector Labs) was done using standard procedures. For evaluation of remyelination brains were fixed with 4% paraformaldehyde, paraffinized, cut into 3-µm thick serial sagittal

sections, deparaffinized and incubated as described above with a polyclonal anti-myelin basic protein (anti-MBP, Biogenex, 1:2) or anti-NF as primary antibodies. Specificity of the stainings was confirmed by omission of the primary antibody.

Electrophysiology and biocytin injections. Sagittal slices of 400 μm thickness were cut on a vibratome and maintained at 34-35°C in an interface-type recording chamber. Artificial cerebrospinal fluid (ACSF) consisted of (in mM) 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1.8 $MgSO_4$, 1.6 $CaCl_2$, 26 $NaHCO_3$ and 10 glucose with a pH of 7.4 when saturated with 95% O_2 - 5% CO_2. Stimuli: 100 μs, 5-20 V were delivered via a bipolar tungsten electrode. Extracellular action potentials were registered with a recording electrode (3-5 MW) located in the middle of the postcommissural fornix. Tetrodotoxin (TTX, Sigma) was applied locally in a concentration of 10 μM (dissolved in ACSF) with a broken micropipette placed on the slice surface near the recording site. Injections of a small biocytin (Sigma) crystal into the fornix were performed with a miniature needle. After an incubation period of 8-10 h in the interface chamber, slices were fixed in 4 % paraformaldehyde, resectioned and reacted with ABC peroxidase reagent (Vector Labs).

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Claims

1. A method for the improvement of neuronal regeneration by prevention or specific inhibition of basal membrane formation induced by a lesion of neuronal tissue.

2. The method according to claim 1, wherein the formation of the basal membrane is prevented or inhibited by applying an inhibitor substance of the synthesis of basal membrane building elements, or the assembly of basal membrane building elements, or both the synthesis of basal membrane building elements and the assembly of basal membrane building elements to a body in need thereof.

3. The method of claim 2, wherein the basal membrane building elements are selected from the group consisting of collagen IV, laminin, entactin, accessory substances for proper function, or the assembly of the basal membrane, or both the proper function and the assembly of the basal membrane.

4. The method of claims 2 and/or 3, wherein the inhibitor substance is selected from the group consisting of antibodies against collagen IV, laminin, entactin, accessory substances for proper function, or the assembly of the basal membrane; Fe-chelating agents; inhibitors of amino acids hydroxylases, such as prolyl-4-hydroxylase, lysine-hydroxylase; 2-oxoglutarate competitors; antisense oligo nucleotides or oligo nucleotide analogs.

5. The method of claim 4, wherein the inhibitor substance is selected from the group consisting of N-oxaloglycine; Zn salts; pyridine derivatives, such as 5-arylcarbonyamino- or 5-arylcarbamoyl-derivatives, 2-carboxylate, 2,5 dicar-

boxylate, their ethyl esters or ethyl amides or -5-acyl sulfonamides, 2,4 dicarboxylate, their ethyl esters or ethylamides, or dimethoxyethylamides; 3,4'-bipyridine, such as 5 amino-6-(1H)-one, 1,6-dihydro-2-methyl-6-oxo-5-carbo-nitril; 2,2'-bipyridine, such as 5,5'-dicarboxylic acid or its pharmaceutically acceptable salts, 4,4'-dicarboxylic acid ethyl ester or ethyl amide; 3,4'-dihydroxybenzoate, such as the diethyl ester; proline and its structural and functional analoges; ß-aminopropionitrile; desferrioxamine; anthracyclines; 2,7,8-trihydroxy anthraquinones, fibrostatin-C; coumalic acid or its pharmaceutically acceptable salts; 5-oxaproline,ß-lactam antibiotics.

6. The method according to any one of the preceding claims, wherein the inhibitor substance(s) are applied in combination with a substance being capable of stimulating neuronal growth or inducing the expression of growth promoting proteins such as fibroblast growth factors, neural cell adhesion molecules like L1 (NILE), growth-associated proteins like GAP43 and anti-apoptotic proteins like bcl-2.

7. The method according to any one of the preceding claims, wherein the inhibitor substances are applied locally in the neuronal tissue, intraventricularly, or systemically.

8. The method according to any one of the preceding claims, wherein the inhibitor substance is applied orally or intravenously.

9. The method according to any one of the preceding claims, wherein the inhibitor substance is applied in therapeutically effective amounts, such as 1 ng/kg to 1 mg/kg body weight.

10. Use of an inhibitor substance which is capable of prevention or specific inhibition of basal membrane formation induced by a lesion of neuronal tissue for the manufacturing of a medicament for the improvement of neuronal regeneration except an inhibitor substance which is an isolated peptide derived from type IV collagen, a peptide inhibiting the laminin nidogen interaction.

11. Use of claim 10 wherein the inhibitor substance is a substance inhibiting the synthesis of basal membrane building elements, or the assembly of basal membrane building elements, or both the synthesis of basal membrane building elements and the assembly of basal membrane building elements.

12. Use of claim 11 wherein the basal membrane building elements are selected from the group consisting of collagen IV, laminin, entactin, accessory substances for proper function, or the assembly of the basal membrane, or both the proper function and the assembly of the basal membrane.

13. Use of claims 10 or 11 wherein the inhibitor substance is selected from the group consisting of antibodies against collagen IV, laminin, entactin, accessory substances for proper function, or the assembly of the basal membrane; Fe-chelating agents; inhibitors of amino acids hydroxylases, such as prolyl-4-hydroxylase, lysine-hydroxylase; 2-oxoglutarate competitors; antisense oligo nucleotides or oligo nucleotide analogs.

14. Use of claim 13 wherein wherein the inhibitor substance is selected from the group consisting of N-oxaloglycine; Zn salts; pyridine derivatives, such as 5-arylcarbonyamino- or 5-arylcarbamoyl-derivatives, 2-carboxylate, 2,5 dicarboxylate, their ethyl esters or ethyl amides or -5-acyl sulfonamides, 2,4 dicarboxylate, their ethyl esters or ethylamides, or dimethoxyethylamides; 3,4'-bipyridine, such as 5 amino-6-(1H)-one, 1,6-dihydro-2-methyl-6-oxo-5-carbo-

nitril; 2,2'-bipyridine, such as 5,5'-dicarboxylic acid or its pharmaceutically acceptable salts, 4,4'-dicarboxylic acid ethyl ester or ethyl amide; 3,4'-dihydroxybenzoate, such as the diethyl ester; proline and its structural and functional analoges; ß-aminopropionitrile; desferrioxamine; anthracyclines; 2,7,8-trihydroxy anthraquinones, fibrostatin-C; coumalic acid or its pharmaceutically acceptable salts; 5-oxaproline,ß-lactam antibiotics.

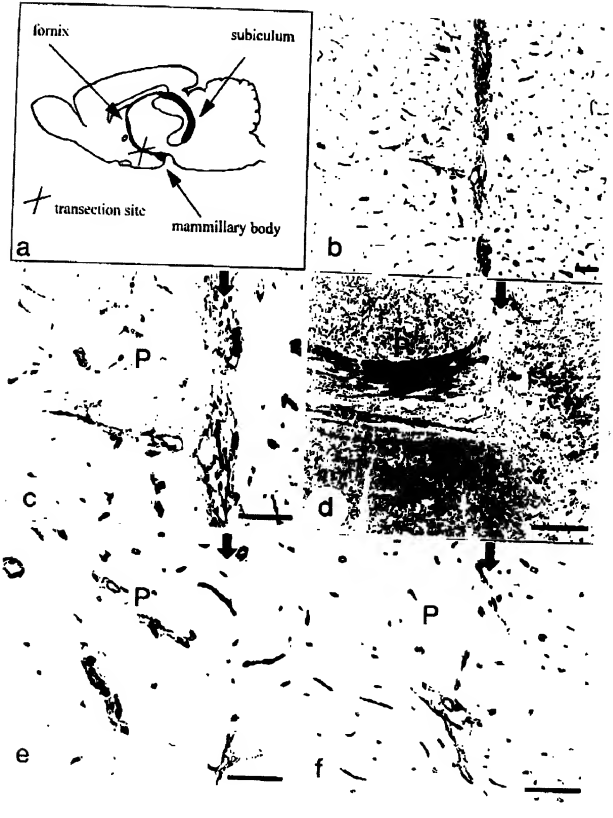
15. A medicament for the improvement of neuronal regeneration comprising a therapeutically effective amount of an inhibitor substance which is capable of prevention or specific inhibition of basal membrane formation induced by a lesion of neuronal tissue comprising the inhibitor substance(s) in combination with a substance being capable of stimulating neuronal growth or inducing the expression of growth promoting proteins such as fibroblast growth factors, neural cell adhesion molecules like L1 (NILE), growth-associated proteins like GAP43 and anti-apoptotic proteins like bcl-2.

16. The medicament according to claim 15 , wherein the inhibitor substance is applied in therapeutically effective amounts, such as 1 ng/kg to 1 mg/kg body weight.

17. Use of a medicament according to claim 15 or 16 for oral or intravenously application or for locally in the neuronal tissue, intraventricularly, or systemically.

Abstract

A method for the improvement of neuronal regeneration by prevention or inhibition of basal membrane formation induced by a lesion of neuronal tissue.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subiculum
transection site
mammillary body
a
b
c
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e
f
P
P
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P

Fig. 1

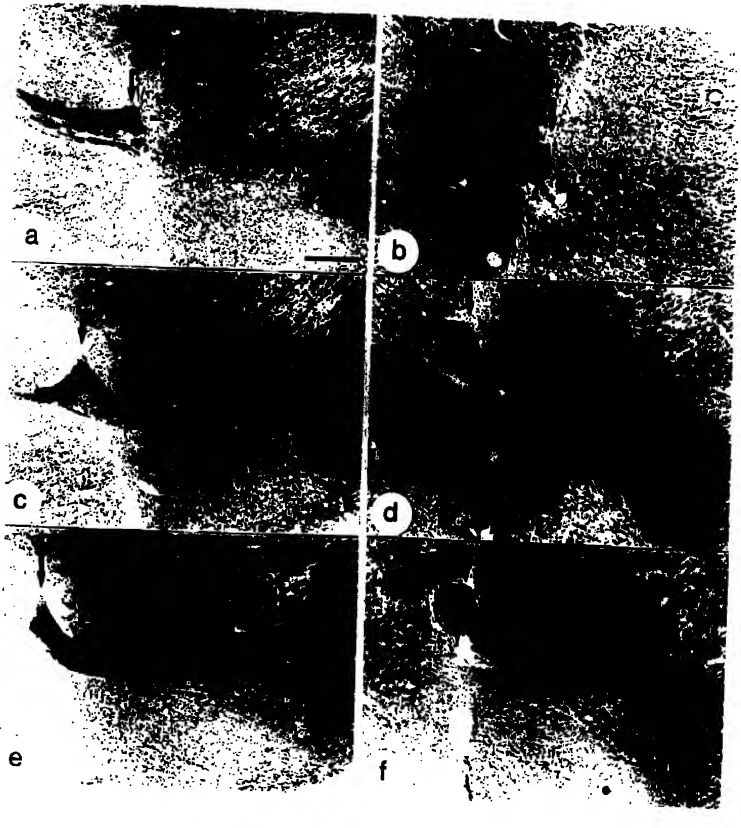

Fig. 2

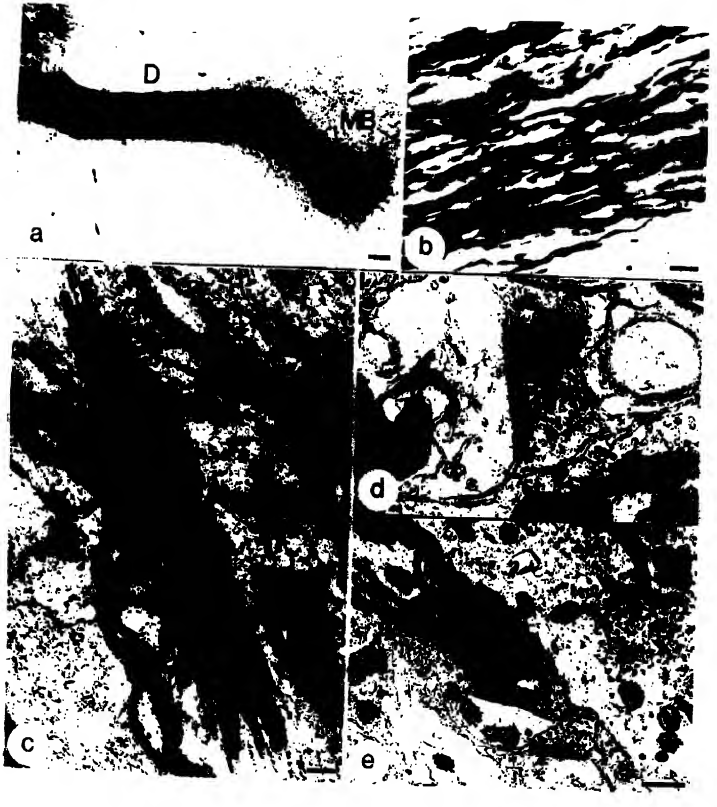

Fig. 3

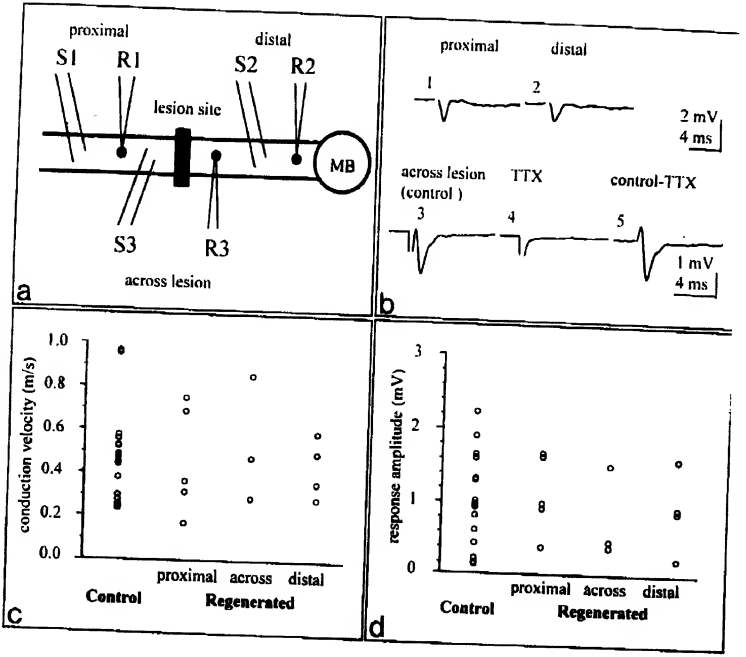
proximal
distal
S1
R1
S2
R2
lesion site
MB
S3
R3
across lesion
a
proximal
distal
1
2
2 mV
4 ms
across lesion
(control)
TTX
control-TTX
3
4
5
1 mV
4 ms
b
conduction velocity (m/s)
1.0
0.8
0.6
0.4
0.2
0.0
proximal across distal
Control
Regenerated
c
response amplitude (mV)
3
2
1
0
proximal across distal
Control
Regenerated
d

Fig. 4

DECLARATION AND POWER OF ATTORNEY U.S.A.

ALL PATENTS, INCLUDING DESIGN
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FOR ATTORNEY'S USE ONLY

ATTORNEY'S DOCKET NO.

P64029US0

As a below named inventor, I declare that my residence, post office address and citizenship are stated below next to my name, the information given herein is true, that I believe that I am the original, first and sole inventor (if only one name is listed at 201 below), or a first and joint inventor (if plural inventors are named below at 201-203, or on additional sheets attached hereto) of the subject matter which is claimed and for which patent is sought on the invention entitled:

101

A METHOD FOR THE IMPROVEMENT OF NEURONAL REGENERATION

102

which is described and claimed in:

☐ the attached specification

☒ PCT International Application No. **PCT/EP98/02808** filed **13 May 1998**

☒ the specification in application (if applicable) and _____ filed **November 15, 1999**

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.
I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.
I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed	
97107846.4	**EP**	**14 May 1997**	☒ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No

103

104

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Application No. _____ Filing Date _____ Application No. _____ Filing Date _____

105

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)

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POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys (Registration No.) to prosecute this application, receive and act on instructions from my agent, and transact all business in the Patent and Trademark Office connected therewith. HARVEY B. JACOBSON JR. (20,851); D. DOUGLAS PRICE (24,514); JOHN CLARKE (31,409)

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	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY	ZIP CODE

I further declare that all statements made herein of my own knowledge are true and that all statement made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 *	SIGNATURE OF INVENTOR 202 *	SIGNATURE OF INVENTOR 203 *
Hans W. Müller	Chr Stichel-Gunkel	
DATE Dec. 21, 1999	DATE Dec. 24, 1999	DATE

☐ Additional inventors are named on separately numbered sheets attached hereto.